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## Brief report

## Contamination in bluetongue virus challenge experiments

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## ABSTRACT

Five cattle and five sheep that had never been exposed to bluetongue virus (BTV), as well as ten animals that had been experimentally infected with BTV-8, were inoculated with BTV-1. Previous exposure to BTV-8 did not prevent a second infection with another serotype.

After the experiment, the BTV-1 preparation was found to be contaminated with BTV-15. The inoculum and blood samples taken during the experiment were analysed by serotype-specific real-time RT-PCR. There was 100-fold less BTV-15 than BTV-1 in the inoculum. Unexpectedly, BTV-15 dominated the infection in cattle that had previously been exposed to BTV-8. In sheep of both groups, on the other hand, BTV-1 prevailed over the contaminant.

Regardless of the outcome, the incident demonstrates the need for a thorough contamination screening of virus preparations. For this purpose, two type-specific RT-PCR primer sets for each of the 24 established BTV serotypes as well as Toggenburg Orbivirus were designed.

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There are at least 24 serotypes of *Bluetongue virus* (BTV), an arthropod-borne reovirus that affects domestic and wild ruminants worldwide. In many research laboratories, different strains and serotypes of BTV as well as related viruses are handled at the same time. Despite best efforts, contamination does occur and can have far-reaching consequences.

In the present study, six Holstein Friesian cattle and four German Blackhead Mutton sheep had been experimentally infected with BTV-8 (isolated in Germany in 2007). Six weeks later, they were challenged with a French isolate of BTV-1, kindly provided by ANSES. To investigate the cross-protective influence of the previous exposure to BTV-8, five BTV-naïve cattle and sheep were also inoculated with the same BTV-1 preparation at the same time. All animals were subcutaneously injected with Vero cell culture supernatant that contained  $10^{6.3}$  TCID<sub>50</sub> of BTV. Since the BTV-1 isolate had originally been intended for PCR validation only, it had not undergone the usual checks for a challenge inoculum at the sending laboratory. In fact, ANSES had not certified it as pure. For the experiment, the received material was passaged once at the FLI. Real-time RT-PCR analysis [1] of the passaged material confirmed

**Table 1**

BTV-15 specific RT-PCR and real-time RT-PCR assays used for sample analysis.

RT-PCR primers	
BTV15-192F	5' GTGTGGACTGAAATATCGTCC 3'
BTV15-604R	5' CAATATGTAACCTACCTCCTTGC 3'
Real-time RT-PCR primers and probe	
BTV15-VP2-978F	5' TGTTCGCCACGGAGAACAGCG 3'
BTV15-VP2-1079R	5' CGGTCGCTGCGATTATTAGAGGTGC 3'
BTV15-VP2-1045FAMas	5' 6FAM-CTGCCTCTGCGTCTCTGCCACTGCCT-BHQ1 3'

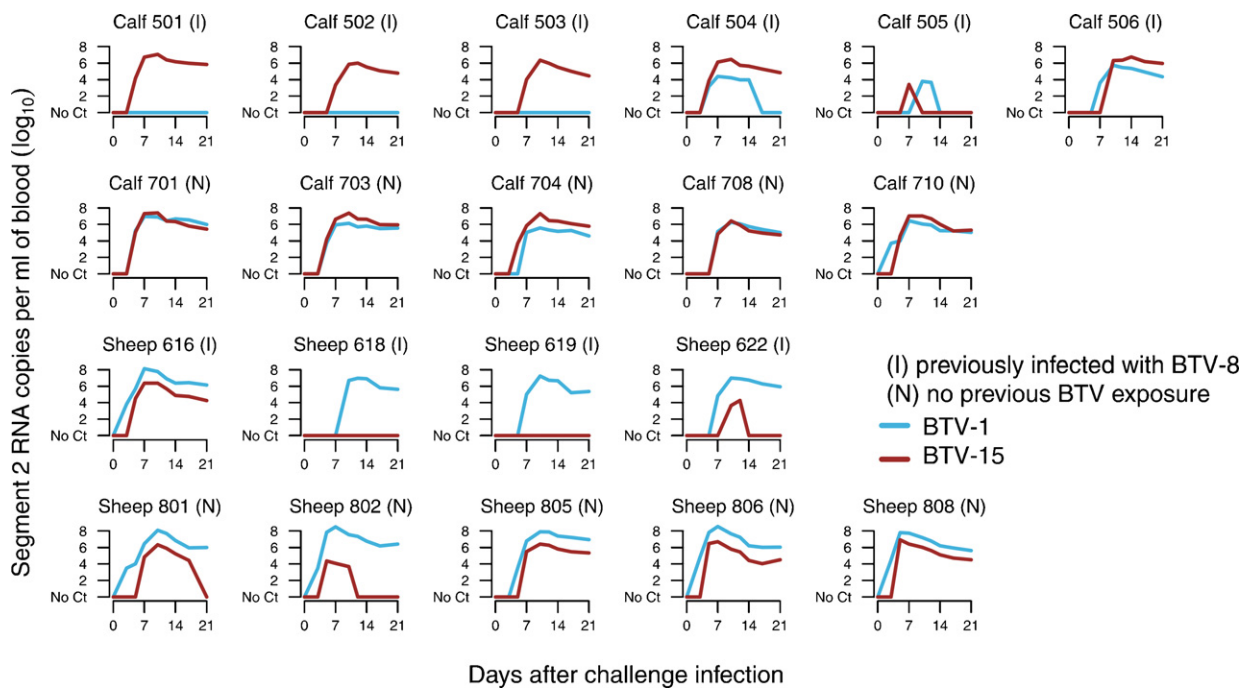
that it contained a large amount of BTV-1, but other serotypes were not tested for. It was later revealed that the BTV-1 isolate had been contaminated with BTV-15.

This contamination was only discovered when samples from the animal experiment were sent to the Istituto G. Caporale for serum neutralization tests. After neutralizing antibodies against BTV-15 had been found in serum samples, blood from the animals and the supernatant used for infection were examined by a serotype-specific RT-PCR amplifying a 413 bp fragment of BTV-15 genome segment 2 (see Table 1). Since these were also positive, the inoculum and all samples from the animal experiment were re-tested using a modified CY5-labeled BTV-1 assay [1] and a newly designed BTV-15 assay (see Table 1) in a single-tube duplex real-time RT-PCR. Serial dilutions of BTV-1 and BTV-15 RNA preparations whose copy numbers had been determined by an externally calibrated pan-BTV S5 assay [2] were used for absolute quantification.

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**Fig. 1.** Results of serotype-specific real-time RT-PCR analysis of blood samples taken after challenge infection.

The VP2 gene sequence of the contaminant was obtained and deposited in GenBank (accession number JF343006). BLAST analysis<sup>2</sup> revealed only 96% identity to the VP2 gene of the South African reference strain “RSArrrr/15”, the only available BTV-15 VP2 gene sequence in the database. The BTV-1 strain used in this study had originally been isolated in France from a sheep with clinical signs of bluetongue. There is no indication that BTV-15 has ever circulated in Europe. An Israeli strain of BTV-15, however, had been handled at the sending laboratory at the same time as the BTV-1 isolate. This is suspected as the origin of the contamination, but no further attempt was made to confirm that.

In the real-time RT-PCR, the inoculum was strongly positive for BTV-1 RNA with a mean  $C_t$  value of 17.1 ( $3.4 \times 10^9$  segment 2 RNA copies per ml), but also contained an about 100-fold lower amount of BTV-15 ( $C_t$  24.6;  $3.6 \times 10^7$  copies per ml). Surprisingly, this difference was not uniformly reflected in the course of infection observed in the experiment.

The outcome was very different between the two host species (see Fig. 1). High amounts of BTV-1 RNA were detected in all sheep, regardless of their BTV exposure history. Two of the four previously BTV-8 infected sheep did not show any BTV-15 replication at all, and it was much reduced in a third. In naïve sheep, BTV-15 replication on average was reduced 100-fold compared to BTV-1.

In three of the six cattle previously infected with BTV-8, on the other hand, no BTV-1 replication was detected at all, and it was about 100-fold reduced in a fourth. Interestingly, previously infected cattle that were negative for BTV-1 were highly positive for BTV-15. Finally, in samples from naïve cattle, BTV-1 and BTV-15 RNA were detected in roughly equal amounts.

In summary, even though there was over 100-fold less BTV-15 in the inoculum, this serotype dominated the infection in cattle that had previously been exposed to BTV-8. In sheep of both groups,

on the other hand, BTV-1 prevailed over the contaminant. A BTV infection with a single serotype generally does not induce immunity to other serotypes [3], and previous BTV-8 exposure did not prevent infection with another BTV serotype in either species in this experiment.

The observed differences in replication suggest that the contaminating strain of BTV-15 is particularly well adapted to cattle, considering it easily made up for the over 100-fold lower inoculated dose. It is unclear whether the dominance of BTV-1 in sheep is simply due to interference between the viruses (where the lower dose could place BTV-15 at a competitive disadvantage), or if BTV-1 in turn is better suited to replication in sheep. Jeggo et al. have previously proposed a mechanism for interference based on cross-reactive cytotoxic T-lymphocytes [4], and another study of mixed BTV infection of sheep has also reported differences in growth behavior between virus strains [5]. It is well established that the clinical manifestation of BTV infection varies between host species as well as virus strains [6], but little is known about the species preference of individual BTV strains, if indeed there is any.

In any case, this experiment as well as a similar incident recently reported by Bréard et al. [7] should serve as a reminder to everybody to always screen their virus preparations carefully. For this purpose, we have prepared two segment 2 primer pairs specific for each of the 24 established BTV serotypes and Toggenburg Orbivirus [8] (see Table 2). Fragment sizes have been reduced compared to previously published primer sets [9] to increase sensitivity; still, owing to the small sample volumes used for nucleic acid extraction, RT-PCR is not always sufficiently sensitive to detect minute contaminations. As the challenge experiment of Bréard et al. aptly demonstrates, the inoculation of animals vaccinated against the predominant serotype is the “gold standard” for contamination screening. Recently, Calvo-Pinilla et al. have shown that vaccination with inactivated vaccines can protect mice against fatal BTV infection [10], providing a small animal model that can be very useful in this context. Our experiment in turn proves the value of a

<sup>2</sup> NCBI Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov>.

**Table 2**  
Degenerate segment 2 RT-PCR primers for all established BTv serotypes and Toggenburg Orbivirus.

Forward primer	Sequence 5'–3'	Reverse primer	Sequence 5'–3'	Fragment sizes (bp)
BTv1-220F	ATGGWWTGTVBTRCCGCGAGT	BTv1-818R	YGTATCCAYCTATTTRCATATCTC	599, 480, 409, 291
BTv1-339F	ATGATGAAAGATTTRATGGACGC	BTv1-629R	CGATCTCTCTKTCAGCGTG	
BTv2-330F	GTWCAGTGGATGATTAARGATAG	BTv2-916R	TCAACSGGTTCCGGCTCTTGTG	587, 540, 383, 336
BTv2-377F	ATCGATTGAYGARGAACATAGCC	BTv2-712R	GTATACGGTTCRTRCCTCCATTG	
BTv3-170F	ATGTAGCCGAGCAAGGCATCA	BTv3-711R	AGCGTTATCTCCTAACTGCATAC	542, 483, 467, 408
BTv3-229F	GAGGTTTACCTCGCATCTTAGA	BTv3-636R	TCCGTTGACAACCGTTCAGA	
BTv4-194F	AGCGCGTAACTATAAACCAAC	BTv4-840R	ATCCGAGTTCTATCATATGACGC	647, 589, 560, 502
BTv4-281F	AAGTCGGTTAAGAACGCACGC	BTv4-782R	TAATYTCYCCYCTTATTATACTCG	
BTv5-280F	AAAGYTTMAACACRAAYAGTGGTGT	BTv5-818R	ACGTTTCCACGCCTCRGTAT	539, 500, 414, 375
BTv5-319F	ATCAYAAATGGATGGARTGGATG	BTv5-693R	TGTCACACYCTTGCYCTTCTCC	
BTv6-168F	GATGTTCCGATTCCGTAAGCG	BTv6-745R	ACTTTGCCCTGCACACGCA	578, 540, 538, 500
BTv6-206F	AAGGAATGATGGTTCAGTGCTA	BTv6-705R	TCCTTTATATGGCTCATCTCTCA	
BTv7-186F	ACCGATGACAGGATGCTAATTC	BTv7-786R	CATCTCTTCTAACCATATCCG	601, 540, 481, 420
BTv7-306F	TATAGAGAAGATGACTAATCAAACC	BTv7-725R	AACATCGCGGTGTACTGGTCT	
BTv8-186F	GATGAACATATACGTGATGCGATTA	BTv8-733R	AACCTCTTCTCAACTCTGGTA	548, 463, 439, 354
BTv8-295F	TTRTTTTGAATGATGGTCATAGCGA	BTv8-648R	AATCACTCGTTGATGCACGTTT	
BTv9-163F	CAATWGATGTGCCWGATGGTGA	BTv9-612R	AATGYACTCKTARWACGTACGTATC	450, 394, 291, 235
BTv9-322F	ATAARTGGATGGARTGGATGCT	BTv9-556R	TTATATAACCCATTATTTATYAGATGGTT	
BTv10-183F	AATCRGATATGATCGACGTACC	BTv10-739R	TTCCGACGGCTGCCCTCTATA	557, 515, 485, 443
BTv10-255F	GWGTGGTYGTGCCGAGATTG	BTv10-697R	CAGCTGTATCCATCTCTGGCT	
BTv11-224F	GAYGGGATCGTGGTCTTAG	BTv11-796R	CWCRTTTAGCCCGCGGATATC	573, 533, 463, 423
BTv11-264F	GAGCKTATGACCATAGAAAAGC	BTv11-686R	TTCCGGTSCCTCTCACTAAG	
BTv12-218F	AATTTATCTCATGTTATAACCCGTC	BTv12-670R	CCCATTACGATGAGTTGATCTC	453, 390, 382, 319
BTv12-281F	CACAAAATTGCGTTAGACAAAACG	BTv12-599R	TCAAAGGTATCCCGCGGCT	
BTv13-177F	GTTTCARCAATAAGAGATGCAAT	BTv13-835R	GCTCATAAATTCCTGTATCCAT	658, 550, 452, 343
BTv13-286F	ATGATGAARAGAGATGGCGTAGA	BTv13-628R	TCTCCGCTCAGAGTTAAACG	
BTv14-169F	ATGTGCCGATACGTGATGCC	BTv14-696R	AAGCATCATACCAGTACCA	528, 457, 439, 368
BTv14-258F	TATGACCGGAGAAATATCACAA	BTv14-625R	CCCTCATCAGTTGTGGCTCT	
BTv15-192F	GTGTTGGACTGAAATATCGTCC	BTv15-670R	CACCTCGCAACTCCTGCTTTG	479, 413, 411, 345
BTv15-260F	ATAAGAGCATACGATCATAAACATC	BTv15-604R	CAATATGTAACCTACCTCCTTGC	
BTv16-187F	ATCAGAGAGGCAATAGAATACAAAAC	BTv16-660R	ATGTTCCGCAATCTAAATTCATC	474, 433, 413, 372
BTv16-248F	AGATATGCTATTGCGTGTACGA	BTv16-619R	CACCAATCACTATAAATCATACGTC	
BTv17-227F	GGRATCGTTGACTAGATTACT	BTv17-689R	CTATTGCGGTTCTCTAGTCAA	463, 386, 355, 278
BTv17-304F	GATAGAGTTCATGACGAACGCTA	BTv17-581R	TAGCACCTTGCAACGCGTG	
BTv18-199F	TATACAAACAGTCAGTAACGATG	BTv18-669R	TCCGATTCAACTCGAGCGTC	471, 416, 385, 329
BTv18-254F	TTTAAGAGCATTTGATGATAGGAAG	BTv18-583R	AATATAAAGCGGCTCCTCG	
BTv19-180F	TGATATACCAATACAGGATGCTAC	BTv19-683R	CCACGCCGATACTAGTTAG	504, 424, 419, 339
BTv19-265F	GCTGCTTACGATACGCGGAA	BTv19-603R	CTTCTCGTAGGCTTGAGCA	
BTv20-204F	ACTATAAACCTGTGAGGAATGATG	BTv20-659R	ATTTCTGGGACCCGATTGGATA	456, 390, 353, 287
BTv20-270F	ACGACGATAGGAAGTCAACGAA	BTv20-556R	GTAAACATCTCAACAGCTGTC	
BTv21-181F	GCGAAGCTCTGAACACTTAC	BTv21-694R	GCATCATCTCTAGAATATTCCG	514, 445, 405, 336
BTv21-250F	TGAGCGGATACGATAGACGA	BTv21-585R	CGAGCGTGGGTTTCAGAATG	
BTv22-156F	AAATTAAGGCGTATGTGTGCGG	BTv22-641R	TTTCCAGCAACTCTGTACGCA	486, 431, 422, 367
BTv22-220F	TTTATCATGTTATCACGCCATC	BTv22-586R	TGCTGTCTTCGATACTGGAA	
BTv23-202F	CTAARCTGTTAGCAATGACG	BTv23-819R	GGCGCYGCTGTAATCCATCT	618, 533, 457, 372
BTv23-287F	AACGAGTGGAGGTCAGAGTG	BTv23-658R	TCTGCTCTCTCCGTTAWAAGT	
BTv24-195F	GACGCACTAGTTACAGACC	BTv24-704R	AGGTTCTCTCTACTTCCTATC	510, 449, 434, 373
BTv24-271F	ATGATCGGAAGCAACGAAGA	BTv24-643R	GGATAACTCTCACCCGTATTCT	
BTv25-193F	CCTTCGAGGAATGATGGGATA	BTv25-698R	AACATATCGTAAGCGTTGCCG	506, 443, 432, 369
BTv25-256F	AAGAGACGGTCGATGAAGGATA	BTv25-624R	GTCATATCTCTGTCGACGTC	

thorough serological analysis of samples beyond the serotypes one expects to be there.

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